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- ☐ 1. 6306588. 06 Feb 98; 23 Oct 01. Polymerases for analyzing or typing polymorphic nucleic acid fragments and uses thereof. Solus; Joseph, et al. 435/6; 435/174 435/91.2 530/350 530/382 530/388.21. C12Q001/68 C12P019/34 C12N011/16 C07K017/00 A61K035/14.
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- ☐ 2. 6110710. 14 Apr 98; 29 Aug 00. Sequence modification of oligonucleotide primers to manipulate non-templated nucleotide addition. Smith; Jeffrey R., et al. 435/91.2; 435/6. C12P019/34 C12Q001/68.
- 
- ☐ 3. 6090590. 10 Aug 99; 18 Jul 00. Reducing nontemplated 3' nucleotide addition to polynucleotide transcripts. Kao; C. Cheng. 435/91.1; 435/6 435/91.2 536/23.1 536/23.72 536/24.3 536/24.33. C12P019/34 C12Q001/68 C07H021/02 C07H021/04.
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- ☐ 4. 6043070. 29 Aug 97; 28 Mar 00. Phosphoramidate-phosphodiester oligonucleotide chimera as primers. Ellis; Nicole M., et al. 435/193; 435/6 435/91.2 436/501 536/22.1 536/24.33. C12N009/10 C07H021/04.
- 
- ☐ 5. 6030813. 16 Mar 99; 29 Feb 00. Phosphoramidate-phosphodiester oligonucleotide chimera utilized as primers. Ellis; Nicole M., et al. 435/91.1; 435/91.2. C12P019/34.
- 
- ☐ 6. WO 9835060 A1, AU 9863251 A, EP 986651 A1, JP 2001511018 W, US 6306588 B1. New mutant polymerases that do not add non-templated 3'-nucleotides - specifically for analysis of hypervariable polymorphisms e.g. for forensic and paternity testing, disease diagnosis. CHATTERJEE, D K, et al. A61K035/14 C07K017/00 C12N001/15 C12N001/19 C12N001/21 C12N005/10 C12N009/12 C12N011/16 C12N015/00 C12N015/09 C12N015/63 C12N015/85 C12P019/34 C12Q001/68.
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L6: Entry 23 of 23

File: USPT

Sep 17, 1996

DOCUMENT-IDENTIFIER: US 5556772 A

TITLE: Polymerase compositions and uses thereof

Brief Summary Paragraph Right (5):

The subject invention provides novel compositions containing a mixture of (a) an enzyme that possesses substantial 3'-5' exonuclease activity and (b) a DNA polymerase with less 3'-5' exonuclease activity than the enzyme possessing substantial 3'-5' exonuclease activity. Preferably, the enzyme with substantial 3'-5' exonuclease activity is a DNA polymerase. Preferably, the DNA polymerase with less 3'-5' exonuclease activity than the enzyme possessing substantial 3'-5' exonuclease activity is a DNA polymerase substantially lacking 3'-5' exonuclease activity. When a step in a technique of interest employing polynucleotide synthesis involves the step of incubation at an elevated temperature, e.g., PCR, both the DNA polymerase and the enzyme with substantial 3'-5' exonuclease activity are thermostable enzymes. A preferred embodiment of the invention is a composition comprising the Taq DNA polymerase (from *Thermus aquaticus*) and the Pfu DNA polymerase (from *Pyrococcus furiosus*).

Brief Summary Paragraph Right (10):

Although compositions comprising a DNA polymerase with less 3'-5' exonuclease activity than the enzyme possessing substantial 3'-5' exonuclease activity may produce superior results in a variety of synthesis experiments, the composition is especially useful in DNA synthesis when there exists one or more mismatched nucleotide(s), particularly mismatches at the 3' end of one or more synthesis primer(s). In such situations, the results achieved, i.e., the amount of synthesis product produced, are significantly greater than the amount of synthesis product obtained using either a DNA polymerase with less 3'-5' exonuclease activity than the enzyme possessing substantial 3'-5' exonuclease activity or with a DNA polymerase possessing substantial 3'-5' exonuclease activity alone. Other advantages of the subject compositions and methods include increased synthesis product yield, increased transcription product length, and the synthesis of polynucleotides that can not be synthesized by a given DNA polymerase alone.

Brief Summary Paragraph Right (12):

DNA polymerases that possess substantial 3'-5' exonuclease activity include the Pfu DNA polymerase, *E. coli* DNA polymerase I, Klenow fragment, T-4 polymerase, T-7 polymerase, *E. coli* DNA pol III, Ultima DNA Polymerase (Cetus), Vent DNA and Deep Vent DNA polymerases (New England Biolabs). When using the subject compositions in reaction mixtures that are exposed to elevated temperatures, e.g., during the PCR technique, use of thermostable DNA polymerases is preferred. Examples of the thermostable DNA polymerases that possess substantial 3'-5' exonuclease activity include Vent DNA polymerase, Ultima DNA polymerase, Deep Vent DNA polymerase, and Pfu DNA polymerases. A particularly preferred DNA polymerase possessing 3'-5' exonuclease activity for use in subject composition is the Pfu DNA polymerase. The Pfu DNA polymerase is commercially available from Stratagene (La Jolla, Calif.). A detailed description of the Pfu DNA polymerase can be found, among other places in U.S. patent application Ser. No. 07/803,627 filed Dec. 2, 1991.

Brief Summary Paragraph Right (14):

When using the subject compositions in reaction mixtures that are exposed to elevated temperatures, e.g., during the PCR technique, use of thermostable DNA polymerases is preferred. The subject composition may also be used with DNA polymerases that have not yet been isolated, provided that the DNA polymerases have less 3'-5' single-stranded DNA exonuclease activity than the enzyme with substantial 3'-5' exonuclease activity

in the subject composition. Assays for both DNA polymerase activity and 3'-5' exonuclease activity can be found in DNA Replication 2nd Ed., Kornberg and Baker, supra, Enzymes, Dixon and Webb, Academic Press, San Diego, Calif. (1979), as well as other publications available to the person of ordinary skill in the art. A preferred DNA polymerase for use in the subject compositions and methods of the invention is the Taq DNA polymerase.

Brief Summary Paragraph Right (16):

The term "thermostable" when used with respect to an enzyme, is readily understood by a person of ordinary skill in the art. Typically, a "thermostable" enzyme retains at least 50 percent of its specific activity after exposure to a temperature of 80.degree. C. for a period of 20 minutes.

Detailed Description Paragraph Right (2):

*Thermus aquaticus* (Taq) DNA polymerase is a 94-kDa protein which does not have an inherent 3' to 5' exonuclease activity (Tindall, K. R., and T. A. Kunkel. 1988. Fidelity of DNA synthesis by the *Thermus aquaticus* DNA polymerase. *Biochemistry* 27:6008-6013). 3' to 5' exonuclease activity enables a polymerase to proofread and is therefore associated with fidelity of an enzyme. The estimated error rate of Taq varies from 2.times.10.sup.-4 mutations per nucleotide per cycle during PCR (Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. 1988. *Science* 239:487-491) to 2.times.10.sup.-5 for nucleotide substitution errors in a single round of DNA synthesis of the lacZ.alpha. gene (Eckert, K. A. and T. A. Kunkel. High fidelity DNA synthesis by the *Thermus aquaticus* DNA polymerase. 1990. *Nucleic Acids Res.* 18:3739-3744). The error rate of Taq DNA polymerase is important in polymerization because it reflects the ability of the polymerase to extend from a mismatched primer:template. Taq DNA polymerase has been shown to extend significantly less efficiently from a mismatched primer: template than from a correctly based paired primer:template (Innis, M. A., K. B. Myambo, D. H. Gelfand and M. A. D. Brow. DNA sequencing with *Thermus aquaticus* DNA polymerase and direct sequencing of polymerase chain reaction-amplification of DNA. 1988. *Proc. Natl. Acad. Sci. USA.* 85:9436-9440 and Kwok, S., D. E. Kellogg, D. Spasic, L. Goda, C. Levenson, and J. J. Sninsky. Effects of primer-template mismatches on the polymerase chain reaction: Human immunodeficiency virus type 1 model studies. 1990. *Nucleic Acids Res.* 18:999-1005).

Detailed Description Paragraph Right (4):

*Pyrococcus furiosus* (Pfu) DNA polymerase is a 91-kDa protein which has an inherent 3' to 5' exonuclease activity. This proofreading activity allows Pfu to extend from mismatched primer:templates by first removing the mismatched base(s) followed by polymerization and results in an error rate of 1.6.times.10.sup.-6 mutations per nucleotide per cycle in PCR reactions. The error rate of Pfu DNA polymerase is thus tenfold lower than that of Taq DNA polymerase and results in higher fidelity (Lundberg, K. S., D. D. Shoemaker, M. W. W. Adams, J. M. Short, J. A. Sorge, and E. J. Mathur. High-fidelity amplification using a thermostable DNA polymerase isolated from *Pyrococcus furiosus*. 1991. *Gene* 108:1-6). The processivity of Pfu DNA polymerase is 10-15 nucleotides per second and its genome is 38% GC.

Detailed Description Paragraph Right (8):

In particular, Taq DNA polymerase requires that 3 to 5 bases at the 3' end of the primer base pair exactly in order for polymerization to occur. The effect of other mismatches on the efficiency of polymerization is dependent on the number of mismatches and where they occur in the primer. This presents a problem when the exact template sequence is not known such as when the nucleotide sequence of the template is derived from amino acid sequence due to the redundancy of the amino acid code and when designing primers for templates of families of genes which are heterogeneous.

Detailed Description Paragraph Right (10):

One of the human heavy chain first constant region primers, AB-61 (Table 15) was chosen for examination. AB-61 has a dGTP as the 3' base and would require a dCTP in the corresponding position in the template in order for efficient priming to occur when Taq DNA polymerase was used for primer extension due to its lack of 3' to 5' exonuclease activity (Kwok, et al. 1990, supra). However, Pfu DNA polymerase does have 3' to 5' exonuclease activity and would remove any mismatched base(s) from the 3' end

of the primer and would therefore be able to extend (Lundberg, et al. 1991, supra). Therefore it was of interest to investigate the effect of using Pfu DNA polymerase alone and in combination with Taq DNA polymerase in primer extension reactions using cDNA from hybridoma cell lines (9F12 and CG7C7) and from human PBLs as the template under various experimental conditions.

Detailed Description Paragraph Right (11):

Kwok, et al. (1990) demonstrated that primer extension efficiency when using Taq DNA polymerase is independent of the dNTP in the template when a dTTP is the 3' base in the primer. Therefore the effect of the addition of one or more dTTPs in various positions at the 3' end of the primer AB-61 (Table 13) was also investigated.

Detailed Description Paragraph Right (26):

These experiments investigated the ability of Taq and Pfu DNA polymerases both together and in separate reactions to extend from primers which contain one or two 3' mismatches. The first experiment demonstrates that Taq DNA polymerase can only extend from a primer which matches at the 3' end under the conditions used (2.1 and 6.1 mM MgCl<sub>2</sub>). The next experiment demonstrates that Taq and Pfu DNA polymerases used in the same reaction will extend from all the primers with 3' mismatches that were used from both hybridoma and PBL templates while neither polymerase alone was able to extend from all primers. The combination of both polymerases also resulted in more product in some of the samples.

Detailed Description Paragraph Right (27):

This series of experiments suggest that Taq in Taq buffer will extend from a primer that is perfectly matched at the 3' end, in V25 buffer will extend from a primer that has one T which creates a mismatch at the 3' end of a primer, and in V25 buffer will not extend from a primer that has two Ts which create two mismatches at the 3' end of a primer. Taq and Pfu DNA polymerases in V25 buffer will extend from a primer that has two Ts which create a mismatch at the 3' end of a primer.

Detailed Description Paragraph Right (29):

The ability of Taq DNA polymerase to extend from primers with one or more mismatches at the 3' end was investigated. Nucleotide sequences of the 5' primers based on the AB-61 primer and used to amplify 9F12 cDNA are given in Table 12. The 3' primer in reactions which amplified the Fd portion of the human heavy chain was AB-76. The 3' primer in reactions which amplified the VH portion of the human heavy chain was MK-39. The light chain 5' VL primer was AB-25 and the 3' CL primer was AB-94.

Detailed Description Paragraph Right (34):

The next experiment investigated the effect of using Pfu and Taq DNA polymerases in the same PCR reaction to extend from primers with 3' matches and mismatches. The same primers were used (MK-205 which has the same 21 bp on the 3' end as AB-61 was substituted for AB-61) as described above to amplify Fd, VH, CH1, and LC from PBL and 9F12 cDNAs. PCR reactions were performed in 1.times. V25 buffer (Table 17) with 200 .mu.M each dNTP and 2.5 units of both Taq and Pfu DNA polymerases or with Pfu DNA polymerase alone using cDNA generated as described above from 9F12 & PBL RNA as the template. Samples were denatured at 94.degree. C. for 5 minutes and annealed at 47.degree. C. for 5 minutes followed by PCR amplification at 71.degree. C. for 3 minutes, 92.degree. C. for 1 minute and 47.degree. C. for 2.5 minutes for 5 cycles and 71.degree. C. for 3 minutes, 92.degree. C. for 1 minute and 51 .degree. C. for 2.5 minutes for 35 cycles. The lower annealing temperatures were used to try to improve primer:template annealing when mismatches occurred. Samples were analyzed as described above and the results are given in Tables 4 and 5.

Detailed Description Paragraph Right (36):

Significant amounts of both Fd and VH PCR products were generated with 9F12 cDNA as a template with all of the 5' primers, including the 3' mismatched primers (AB-714 to AB-717), when both Taq and Pfu DNA polymerases were used. This contrasted with the previous experiment when Taq DNA polymerase was not able to extend from the 3' mismatched primers (AB-714 through AB-717) under the conditions used. These results suggested that Pfu DNA polymerase can remove 1-2 mismatched bases from the 3' end of the primer. This would enable either Pfu or Taq DNA polymerase to extend from the perfectly base paired primer:template. Taq and Pfu DNA polymerases with 9F12 and PBL templates

Detailed Description Paragraph Right (40):

In this experiment Taq DNA polymerase was able to extend from the 3' mismatched primers which contain only a single mismatched base (AB-714, AB-716, and AB-717) but not one that has two mismatched bases (AB-715) with both the PBL and 9F 12 cDNA templates. In a previous experiment, Taq DNA polymerase was not able to do this, however, these results are consistent with the results of Kwok, et al. (1990, supra). Both AB-715 and AB-717 contain 2 dTTPs at the 3' end of the primer but in different positions. AB-715 replaces the last two dGTPs on the 3' end and AB-717 replaces the last dGTP and adds a dTTP to the 3' end. Because the corresponding base in the template is not known, the 3' dTTP in AB-717 may not create a mismatch with the template.

Detailed Description Paragraph Right (53):

Results indicated that none of the polymerases or buffer combinations tested extended from the AB-715 primer. AB-61 and AB-715 differ by the deletion of two dGTPs and the addition of two dTTPs at the 3' end (Table 13). The lower annealing temperatures did produce either the presence of or an increase in the amount of PCR product generated.

Detailed Description Paragraph Right (59):

Seven different templates with eight different primer sets were used in this experiment. The genomic DNAs were isolated from human, Epstein Barr virus, Escherichia coli, and transgenic and normal mouse. Plasmid DNAs were pBluescript II and pBluescript containing the light chain and Fd of an anti-tetanus toxoid immunoglobulin (Mullinax et al., 1990, supra). Primer sequences and their respective DNA templates are given in Table 16. The thermostable polymerases used in this experiment were Taq, Exo.sup.+ and Exo.sup.- Pfu, and ES-4 DNA polymerases (Stratagene; La Jolla, Calif.) at final concentrations of 2.5, 1.25, 1.25, and 0.125 units per reaction, respectively. PCR reactions were at a primer concentration of 0.2 .mu.M of each 5' and 3' primer in 1.times. T buffer, 1.times. Pfu #1, or 1.times. Pfu #3 (Table 17) with 200 .mu.M each dNTP. An additional reaction with Taq DNA polymerase in 1.times. Taq buffer was also performed. Samples were denatured at 95.degree. C. for 5 minutes and annealed at 40.degree. C. for 5 minutes followed by PCR amplification at 95.degree. C. for 0.5 minutes, 40.degree. C. for 1.5 minutes and 68.degree. C. for 3 minutes for 25 cycles. Samples were analyzed as previously described and results are not given.

Other Reference Publication (4):

Clark, James M. "Novel non-templated nucleotide addition reactions catalyzed by procaryotic and eucaryotic DNA polymerases". Nucleic Acids Research 16:9677-9686 (1988).

Other Reference Publication (9):

Hu, Gengxi. "DNA Polymerase-Catalyzed Addition of Nontemplated Extra Nucleotides to the 3' End of a DNA Fragment". DNA and Cell Biology 12(8):763-770 (1993).

Other Reference Publication (15):

Nassal, Michael and Rieger, Andrea. "PCR-based site-directed mutagenesis using primers with mismatched 3'-ends".

CLAIMS:

2. A kit according to claim 1, wherein said first and second DNA polymerases are thermostable.

4. A method according to claim 3 wherein said first and second DNA polymerases are thermostable.